Covert infections with a honeybee virus induce fitness costs in ant colonies

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Abstract:

Acute Bee Paralysis Virus (ABPV) is a key driver of honey bee colony losses that has been increasingly reported in non-bee hosts in the past decades. Ants have long been hypothesised to act as viral reservoirs, but most evidence comes from field surveys, and experimental tests are still scarce. Here, we combined survival and transmission assays, viral load measurements, viral replication assays, and host immune gene expression analyses to test whether the clonal raider ant *Ooceraea biroi* can harbour and transmit ABPV. ABPV-injected ants showed delayed development and elevated mortality. Although we found no evidence of viral replication, the virus was able to spread among colony members and viral particles persisted in colonies for several days. These results show that ants can acquire ABPV, incur fitness costs, and pass the virus within their colonies, suggesting that they may act as incidental viral reservoirs. By maintaining and disseminating honey bee viruses, even without supporting replication, ants could contribute to their environmental persistence and spillover across species.

1. Introduction

Honey bees are vital pollinators for ecosystems and agriculture, yet highly vulnerable to human activities and environmental changes. Over the past two decades, substantial winter colony losses of western honeybees, *Apis mellifera*, have been reported worldwide [1]. Several iflaviruses and dicistroviruses have emerged as major drivers of colony losses in both managed and wild populations [1–5]. While these viruses can persist covertly at low titres without causing apparent symptoms, the introduction of the ectoparasitic mite *Varroa destructor* (henceforth: varroa) has facilitated viral spread and turned asymptomatic infections into lethal diseases [6] by injecting viruses into the bee haemolymph [7].

Acute Bee Paralysis Virus (ABPV), a +ssRNA virus in the family *Dicistroviridae* [8,9], has been detected repeatedly in collapsing and *Varroa*-infested colonies and is considered a significant driver of honey bee mortality [9–14]. It occurs across all host life stages and castes, with pupae being especially permissive to viral replication [9,14–16]. Experimental infections have shown that the injection of fewer than 100 viral particles into pupae or adults can induce tremors, paralysis, and death within days (Bailey et al., 1963). ABPV has been detected in bumble bees [17–20] and ants [21–27] and is considered a multi-host virus, with cross-species transmission facilitated by shared floral resources and nesting sites [5].

In recent years, ants have been proposed as viral reservoirs for ABPV based on repeated field detections [21–26,28]. With an estimated 20 quadrillion individuals and a biomass exceeding that of all wild birds and mammals, ants are globally dominant insects [29,30] whose foraging and nesting ranges overlap with those of many taxa, making them potential pathogen vectors [31,32]. ABPV has been reported in ants and honey bees from ant-infested hives, suggesting ants as viral vectors within apiaries [28]. Yet, most studies remain observational, and whether ants can sustain viral replication, transmit infections within colonies, or mount effective immune responses is largely unknown. Experimental work is limited to one study, which showed that oral exposure to ABPV impaired locomotion and colony growth in *Lasius niger* [33].

Here, we investigate ABPV infections in the clonal raider ant *Ooceraea biroi*, a powerful model for studying disease dynamics under controlled laboratory conditions. Workers in *O. biroi* colonies reproduce clonally and synchronously [34], allowing precise experimental control over genotype and age at the individual and colony levels. In addition, the predatory lifestyle of *O. biroi*, whose diet includes ants and other insects [35], makes them prone to pathogen spillovers.

To investigate the consequences of ABPV exposure in this system, we measured the effects of controlled inoculations on host survival, assessed the ability of the virus to replicate within hosts and transmit within colonies, and measured host immune responses by quantifying antiviral immune gene expression [36–39].

2. Materials & Methods

(a) Ant rearing

All ants were sourced from stock colonies of genetic lineage B [40], and maintained at 28 °C in airtight plastic containers (8 x 12 x 5 cm) with a humidified plaster of Paris floor (Brand). The nests were regularly cleaned, re-humidified, and fed with frozen ant brood (*Tetramorium bicarinatum* and *Oecophylla* sp.), as well as frozen houseflies. For each experiment, all ants were clonally related.

(b) Infection assay in pupae and adults

(i) Preparation of viral inoculum

The ABPV inoculum was prepared as described by Tehel et al. (2019). Briefly, 1 µL of homogenate from a heavily ABPV-infected *A. mellifera* worker was injected into white-eyed pupae obtained from virus-free colonies. The injected pupae were incubated for three to five days to allow viral replication and systemic infection, after which they were homogenised in 0.5 M cold potassium phosphate buffer (PPB, pH 8.0). The resulting homogenates were screened using RT-qPCR to confirm the absence of other common bee viruses, such as deformed wing virus (DWV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), and slow bee paralysis virus (SBPV). Only homogenates confirmed to contain ABPV exclusively were used in the experiments. Control inoculum was prepared from uninjected white-eyed pupae processed in the same way.

(ii) Inoculation via microinjection

10-day old ant pupae were placed on glass slides and injected with viral inoculum (ABPV at 10^5 genome equivalents, GEs) laterally at the posterior abdomen using a FemtoJet microinjector (Pi = 200, Pc = 30; Eppendorf AG, Hamburg, Germany) and borosilicate glass capillaries (8.89 cm length, 0.530 mm ± 25 µm inner diameter, 1.14 mm outer diameter; World Precision Instruments). Inoculum was injected until the pupal body visibly stretched. Control pupae were injected with the control inoculum following the same procedure. Following inoculation, eight pupae of either treatment were placed together with six paint-marked, uninjected cohabiting adults in plastic cups (diameter 4.5 cm) with a humidified plaster of Paris floor. For each treatment (control and ABPV-injected) and each sampling time point (0, 3, and 5 days post-inoculation, d.p.i.), four replicate colonies were established. At time point 0, pupae were flash-frozen immediately after injection. At later time points (3 and 5 d.p.i.). Both pupae and cohabiting adults were frozen to assess viral transmission. Pupae survival and development were assessed in the colonies sampled 5 d.p.i. (four control and four infected colonies). Pupae that disappeared, turned dark, or were partly cannibalized were classified as dead.

Adult ants (mixed age) were immobilised by placing them in slits cut in pencil erasers (Läufer Plast 0120) and injected with 10 nL of viral inoculum (ABPV 5×10^4 GEs) or control inoculum at a rate of 5 nL/s under the largest abdominal tergite using a NanoFil 36 GA bevelled needle connected to a NanoFil syringe and an electronic microinjection pump (UMP3 pump with Micro2T SMARTouch controller, World Precision Instruments, USA). Ten injected adults from either treatment were housed with five paint-marked, uninjected cohabiting adults in plastic cups (diameter 4.5 cm) with a humidified plaster of Paris floor. For each treatment (control and ABPV-injected) and each sampling time point (0, 1, 3, and 5 d.p.i.), four replicate colonies were established. At time point 0, focal ants were flash-frozen immediately after the injection. At later time points (1, 3, and 5 d.p.i.), both focal ants and cohabiting adults were flash-frozen. The colonies sampled 5 d.p.i. were monitored for survival over the full period before sampling.

(c) Viral load quantification

For each colony, five injected adults or three injected pupae were flash-frozen in liquid nitrogen and stored at -80 °C. In the ABPV-injected groups, five cohabiting adults were also sampled. Total RNA was extracted from pooled, whole-body samples using a combined Trizol/phenol-chloroform-column protocol [41] and eluted in 20 µL of nuclease-free water. RNA concentration and purity were assessed using a BioPhotometer D30 (Eppendorf, Germany).

Viral load quantification was performed according to a protocol modified from Tehel et al. (2019). For cDNA synthesis, 200 ng of total RNA was reverse-transcribed using oligo(dT)18 primers (Thermo Scientific) and reverse transcriptase (M-MLV and Revertase, Promega, Mannheim, Germany) according to the manufacturer's instructions in a Veriti Thermal Cycler (Applied Biosystems). The resulting cDNA was diluted 1:3 and stored at -20 °C. qPCR was performed using the KAPA SYBR FAST Universal kit (Kapa Biosystems, Sigma-Aldrich) on a LightCycler 480 (Roche), with two technical replicates per sample, and the following thermal cycle: three min at 95 °C; 40 cycles of 10 s at 95 °C, 30 s at 57 °C, 20 s at 72 °C; final elongation for 1 min at 95 °C; melt curve analysis was performed from 55 °C to 95 °C with 0.5 °C per second increments to check for amplification specificity. Forward primer (5′-CATGGCTCAAGACACTTCATCG-3′) and reverse primer (5′-CCAGCAATGACCTCAATGTG G-3′) were used to amplify a 105 bp fragment specific to ABPV capsid protein gene, were designed with Primer-BLAST [42]. Each plate included negative controls (control inoculum and no-template control) and a positive control (viral inoculum). Samples with Ct values >35 or with high variation between technical duplicates (Ct standard deviation >0.5) were re-assayed, except for negative controls and control inoculum, with actin (host reference gene) amplified using primers Ob-actinF (5'-GCCGATCGTATGCAGAAGGA-3') and Ob-actinR (5'-ACCCTTGGCGATGTGTCTTT-3') designed with Primer-BLAST. To quantify the number of viral GEs in each sample, external DNA standards were generated from the viral inoculum using primers ABPV-F6531 (5′-TCATACCTGCCGATCAAG-3′) (Locke et al., 2012) and ABPV-R6774 (5'- CCAGCAATGACCTCAATGTGG-3'). The PCR product size (244 bp) was verified by electrophoresis on a 2% agarose gel, and the corresponding band was excised and purified using the NucleoSpin Gel and PCR Purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified PCR product was quantified using a spectrophotometre and used to generate ten-fold serial dilutions. Viral loads were expressed as $\log_{10}$ GEs per individual ant by dividing total GEs by the number of individuals in each sample.

(d) Negative-sense strand detection

In a separate experiment, three-week-old adult ants were injected with 10 nL of viral inoculum (ABPV 10^4 GEs), control inoculum, or left uninjected; five focal adults from each treatment were housed with five paint-marked, uninjected cohabiting adults, as described above. A lower dose was chosen to increase survival and extend the observation period. For each treatment and time point (0, 6, 12, 24, and 48 hours post-injection, h.p.i.), four replicate colonies were established. At each time point, focal and cohabiting ants from each replicate were pooled separately, flash-frozen in liquid nitrogen, and stored at -80 °C. RNA was extracted as described above, and the extracted RNA was divided, with a portion used for the negative-strand assay and the remaining portion used to quantify immune gene expression (see below).

Focal and cohabiting ants sampled 24 h.p.i were used to assess viral replication via negative-strand detection. This method relies on RT-qPCR amplification of negative-strand RNA intermediates, which serve as markers of active replication of positive-strand RNA viruses within host cells, rather than the presence of inert genomic RNA [9,43]. Two-step RT-PCR was performed using a tagged forward primer designed to specifically anneal to the complementary DNA (cDNA) product of the negative-sense RNA strand of ABPV. Superscript III reverse transcriptase (Invitrogen, USA) was used for cDNA synthesis. In preliminary tests, all ant and bee samples produced non-specific PCR products using previously published primers (Schläppi et al., 2020). To avoid false positives, we designed new primers for the

assay. A total of 200 ng RNA was diluted to 10 µL with water and combined with 1 µL of the tagged forward primer tag-ABPV(-)F5185 (5'-AGCCTGCGCACCGTGG-TTGGTTTGGTGCA GAAGGTG-3') and 1 µL 10 mM dNTPs. The mixture was incubated at 65 °C for 5 min and then placed on ice for at least 1 min. Subsequently, 4 µL of 5X First-Strand Buffer, 1 µL of 0.1 M DTT, and 1 µL of Superscript III Reverse Transcriptase were added, bringing the total reaction volume to 20 µL. The reaction was incubated at 55 °C for 50 min, followed by incubation at 70 °C for 15 min to inactivate the enzyme. cDNA was purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and eluted in 20 µL of elution buffer. The purified cDNA (diluted 1:3 with nuclease-free water) served as the template for PCR amplification with Taq polymerase (New England Biolabs), using a tag oligonucleotide (5'-AGCCTGCGCACCGTGG-3') as the forward primer and the corresponding reverse primer, ABPV(-)R5380 (5'-AGAAAAGTCCATAGGCCCGT-3'). PCR reactions without the tag primer were performed for all samples to verify the complete removal of residual tagged forward primers using the following cycling conditions: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 20 s, and extension at 68 °C for 30 s; and final extension at 68 °C for 2 min. The resulting PCR products were analysed by electrophoresis on a 2% agarose gel. A 196 bp PCR product was expected in samples containing negative-sense RNA, indicating active viral replication. Bee pupae injected with control inoculum or ABPV inoculum served as negative and positive controls, respectively.

(e) Immune gene expression

RNA extracted from adults injected with 10^4 GEs ABPV, which was also used for the negative-strand assay, was used to quantify the expression of the immune gene *Dcr-2* in ABPV-injected ants, control-injected ants, uninjected ants, and cohabiting ants. qPCR were performed as for viral load quantification, with different primers. Primers specific for *Dcr-2* were designed using Primer-BLAST: Dcr-2F (5′-CTCGGTGGATCGGCGAAATA-3′) and Dcr-2R (5′-TGAGGTTCACGATCATCGCC-3′), and were used for qPCR analysis. Relative expression levels were normalised to the housekeeping gene ribosomal protein L13a (RPL-13a), amplified using primers RPL-13aF (5′-GCAAACAAAAGCGTGTCAAG-3′) and RPL-13aR (5′-TCGGCCAGAGTAAAACCTCT-3′) [44].

(f) Statistical analyses

All data analyses were performed using R version 4.4.1 (R Core Team, 2024). Models were selected based on Akaike's Information Criterion (AIC) [45] and compared using anova() function to test if added complexity significantly improved model fit. The final model assumptions and residual diagnostics were assessed using the *DHARMa* package [46]. Post-hoc pairwise comparisons were performed with the *emmeans* package [47] with Tukey's adjustment for multiple comparisons.

<u>Survival</u>

Due to complete separation in the pupal development data (no deaths in the control group and no eclosion in the ABPV-inoculated group), Wilcoxon-Mann-Whitney tests were used to compare the proportion of pupae that hatched into adults or died between treatment groups. For adult survival, the data violated the proportional hazard assumption, and endpoint mortality was therefore analysed using a generalised linear model (GLM) with a binomial error structure and logit link, with Treatment as a fixed factor and Colony as a random factor.

<u>Viral load</u>

The viral load in focal and cohabiting ants was quantified by converting the Ct values to GEs using a standard curve, followed by a $\log_{10}$ transformation. These transformed titres were analysed using a linear model (*lm* function, base R) with one focal and one cohabiting sample per colony. To assess how viral load changed over time in focal individuals, models included Treatment (control-injected vs. ABPV-injected) and Timepoint (0, 3, and 5 d.p.i. for pupae; 0, 1, 3, and 5 d.p.i. for adults), and their interaction (Treatment × Timepoint) as fixed

effects. To examine the viral load differences between focal and cohabiting individuals, a separate model was fitted for overlapping timepoints between the two groups including Individual type (focal vs. cohabiting) as a fixed factor, along with Treatment and Timepoint. For pupae infection assay, comparisons between focal and cohabiting individuals were performed only within the virus-inoculated treatment group as cohabiting adults were only sampled in colonies of virus-inoculated pupae; thus, only Individual type and Timepoint were included as fixed effects.

Immune gene expression

For visualisation, *Dcr-2* expression was normalised to the reference gene (*RPL-13a*), and fold change ($2^{-\Delta\Delta Cq}$) relative to uninjected controls was calculated for injected focal ants and cohabiting ants from control and ABPV-injected groups. The effects of Treatment (control-injected vs. ABPV-injected), Time point (0, 12, 24 and 48 h.p.i.) were analyzed separately for focal and cohabitating individuals using linear models (*lm* function, base R) on $\log_2$-transformed −ΔΔCq values as each sample was a pooled ants from the single colony.

3. Results

ABPV-injected ant pupae had drastically reduced eclosion rates compared to control-injected pupae 5 d.p.i. (mean ± SD: 0 ± 0% in ABPV, 75 ± 10.2% in controls; Wilcoxon–Mann–Whitney test: z = 2.477, p = 0.0286; Figure 1A). Similarly, ABPV-injected pupae had higher mortality compared to controls (mean ± SD: 31.3 ± 16.1% mortality in ABPV, 0 ± 0% mortality in controls; Wilcoxon–Mann–Whitney test; z = -2.461, p-value = 0.0286).

ABPV-injected adult ants had higher mortality than control-injected adults (mean ± SD: 47.5 ± 31% in ABPV; 15 ± 12.9% in controls; $\chi^2(1) = 4.628$, p = 0.031), a 5.1-fold increase in the odds of death, mostly occurring 1 d.p.i (Figure 1B). Although virus-injected focal pupae and adults showed increased mortality, no deaths were observed among the cohabiting adults.

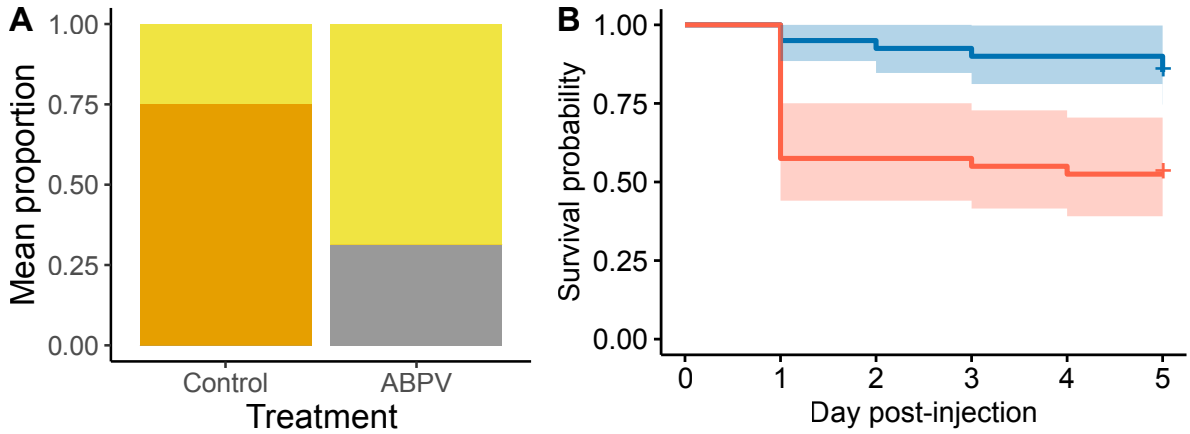

Figure 1. ABPV affects *O. biroi* development and survival. (A) Development and survival of ABPV-injected (10^5 GEs, n=4 colonies) and control-injected pupae (n=4 colonies), shown as the mean proportion of pupae (yellow) that eclosed into adults (orange) or died (gray) five days post-injection. **(B)** Survival of ABPV-injected (5×10^4 GEs, red, n=40 individuals) and control-injected (blue) adult ants (n=40 individuals). Kaplan–Meier curves with 95% confidence intervals.

Viral load differed between ABPV-injected and control-injected ant pupae (ANOVA: $F_{1,18}$ = 51.802, p < 0.001) and across time points ($F_{2,18}$ = 5.207, p = 0.016) (Figure 2A). As expected, ABPV-injected pupae had higher viral loads than control-injected pupae at all time points (Tukey's post-hoc; Control vs. ABPV at 0 d.p.i.: t = -7.197, p < 0.0001; 3 d.p.i.: t = -5.726, p <.0001; 5 d.p.i: t = -3.500, p = 0.003). In ABPV-injected pupae, viral load remained consistently high up to 5 d.p.i. (Tukey's post-hoc; 0 vs. 5 d.p.i.: t = 1.2237, p = 0.447).

In adult ants, viral differed between treatments (ANOVA: $F_{1,16}$ = 140.252, p < 0.001) the difference between the control and ABPV-injected groups was not constant across all time points (ANOVA; treatment × timepoint interaction: $F_{3,16}$ = 3.87, p = 0.030, Figure 2B). This effect was driven by a sharp decline in viral load in ABPV-injected adults within the first day post-injection (Tukey's post-hoc; 0 vs. 1 d.p.i.: t = 4.306, p = 0.003), decreasing from approximately $10^{3.08}$ to $10^{1.42}$ GEs per individual. Thereafter, viral titres remained at a similar level up to day 5 (Tukey's post-hoc; 1 vs. 5 d.p.i.: t = 0.795, p = 0.856).

We consistently detected ABPV in uninjected adults that had cohabited with ABPV-injected pupae (Figure 2A) and adults (Figure 2B) for three to five days. Strikingly, adults cohabiting

with ABPV-injected pupae carried viral loads as high as those of the injected pupae themselves (ANOVA; focal pupae vs. cohabiting adults, $F_{1, 13}$= 1.922, p = 0.189), suggesting efficient transmission from pupae to adults. In contrast, adults cohabiting with ABPV-injected adults had viral loads lower than those in the injected adults themselves (Tukey's post-hoc; cohabiting vs. focal: t = -5.381, p <0.0001), yet they still had significantly higher viral loads than controls (Tukey's post-hoc; control-cohabiting vs. ABPV-cohabiting: t = -6.615, p < 0.0001). Thus, this data indicates rapid and efficient horizontal transmission of the virus, both from the brood to adults and between adults.

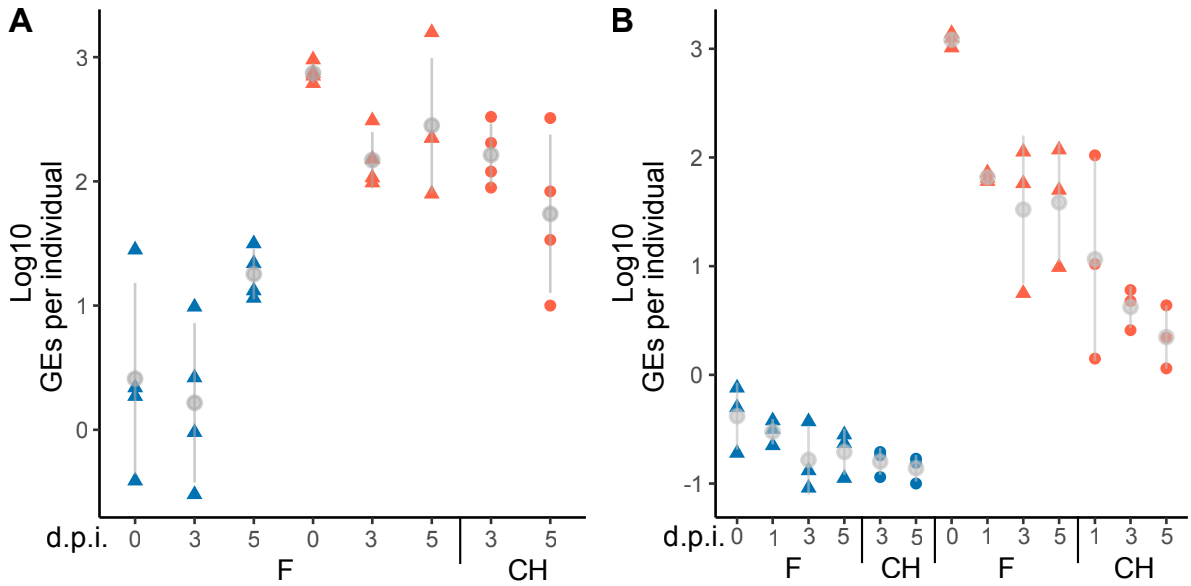

Figure 2. ABPV transmits within ant colonies. (A) Viral load in control-injected pupae (blue triangles), ABPV-injected pupae (red triangles), and cohabiting adults (red circles). Data points represent pools of three pupae or five cohabiting adults. Grey: mean ± SD. **(B)** Viral load in control-injected adults (blue triangles), ABPV-injected adults (red triangles), and cohabiting adults (blue and red circles). Data points represent pools of five adults (n = 3-4 per treatment and time point). d.p.i.: days post-injection. Grey: mean ± SD.

In line with the lack of increase in viral load over time, we found no evidence of viral replication in adult ants. Viral negative-strand replication intermediates were detected in positive controls (ABPV-injected honey bee pupae), but not in ABPV-injected, control-injected, and cohabiting adult ants 24 h post-injection (Figure 3).

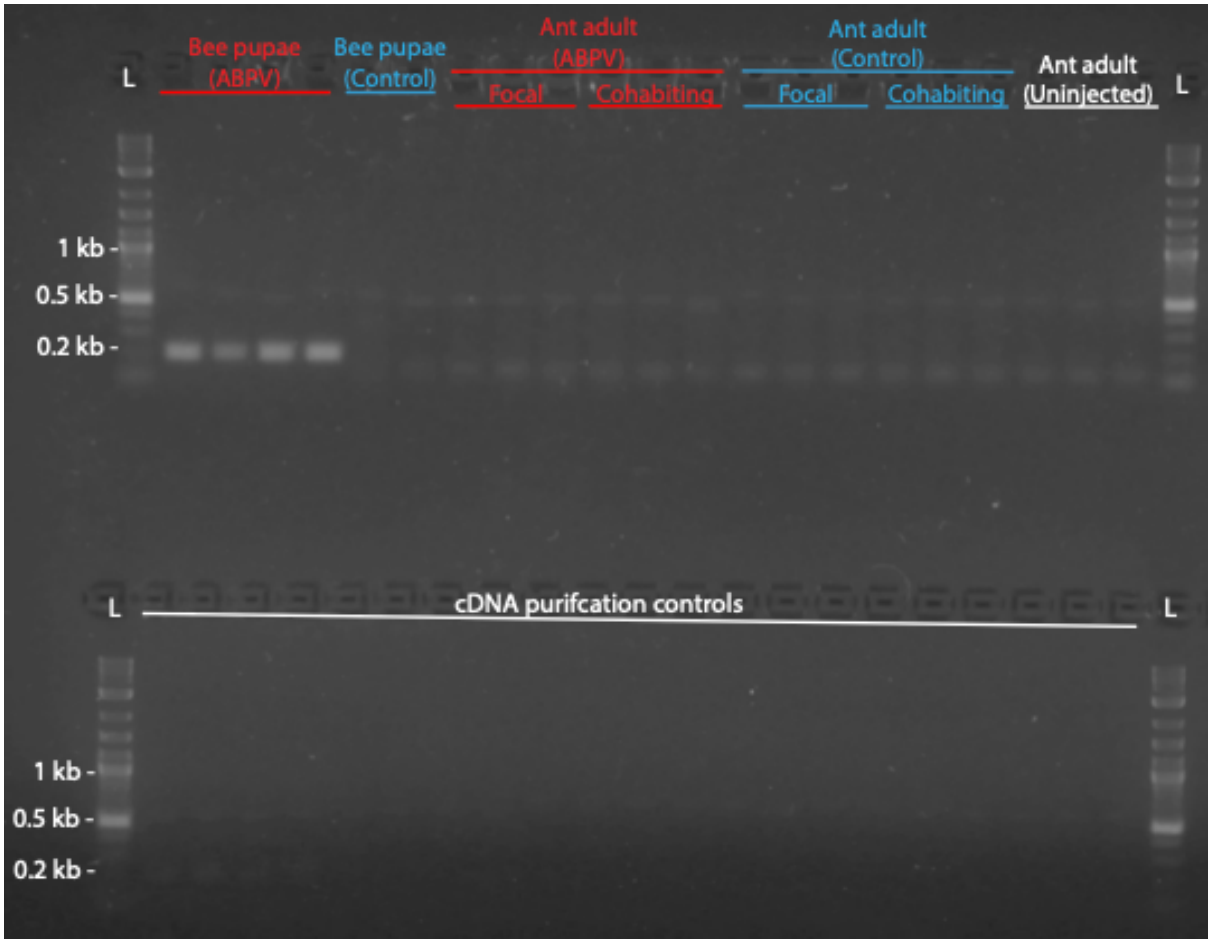

Figure 3. ABPV does not replicate in adult ants. Top row: ABPV-injected and control-injected bee pupae served as positive and negative controls, respectively. Red: ABPV-injected focals and cohabiting ants; blue: control-injected focals and cohabiting ants; white: uninjected. The expected PCR product size was 196 bp. L: DNA ladder. Bottom row: cDNA purification controls corresponding to the samples in the top row.

We investigated host immune responses by measuring the expression of the antiviral gene *Dcr-2* in response to ABPV injection. In focal ants, *Dcr-2* expression changed with time (ANOVA; time point: $F_{3,35}$= 4.759, p = 0.007; Figure 4A) regardless of the treatment (Treatment: F1,35 = 0.030, p= 0.864). Similarly, in cohabiting adults, *Dcr-2* gene expression varied with time (ANOVA; time point: $F_{2,27}$= 23.134, p <0.001) but not with treatment (Figure 4B; ANOVA; Treatment: F1,27 = 2.7126, p= 0.111). Thus, *Dcr-2* did not respond to ABPV inoculation under the conditions tested here.

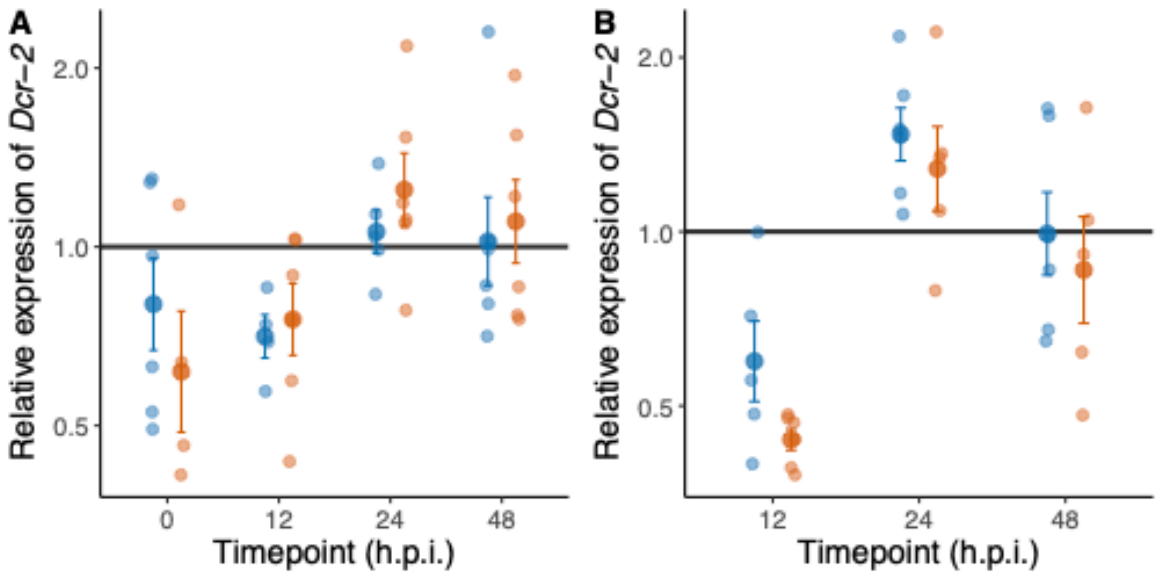

Figure 4. The expression of *Dcr-2* is not affected by ABPV injection. *Dcr-2* expression over time (hours post-injection, h.p.i.) in (A) injected and (B) cohabiting ants. Expression levels are normalised to the reference gene (RPL-13a) and presented as fold changes relative to uninjected ants. Data points represent pools of five adults; orange: ABPV-injected and cohabiting adults; blue: control-injected and cohabiting adults. Large circles and error bars represent the mean ± SEM. The horizontal line at 1 indicates the expression level of *Dcr-2* in uninjected ants.

4. Discussion

Our study shows that injection of ABPV into *O. biroi* pupae and adults reduced host fitness by delaying development and decreasing survival. ABPV persisted over days in inoculated adults and pupae and was efficiently transmitted to adults, likely through social interactions or the nest environment. Strikingly, uninoculated nestmates acquired ABPV titres comparable to those of directly inoculated ants. These results demonstrate that ABPV particles can persist in ants, spread among nestmates, and impact host fitness, even in the absence of detectable viral replication or canonical antiviral RNAi response, as indicated by stable *Dcr-2* expression.

The higher mortality observed in *O. biroi* injected with ABPV is in line with previous studies demonstrating that bee viruses such as ABPV, can impair fitness in non-honey bee hosts, with outcomes depending on the inoculation methods and dosage. For example, black garden ants (*Lasius niger*) fed on ABPV-infected bee pupae for ten weeks showed impaired locomotion and reduced adult emergence [33]. Similarly, IAPV, a close relative of ABPV [9], reduces host reproductive success and survival when fed to bumblebees at very high doses (2×10^7 and 1×10^8 particles), [38,48,49], while relatively low-dose injections of IAPV (ca. 10^2 particles) were sufficient to cause acute infection, paralysis, and mortality in bumblebee workers [50,51].

Despite host fitness impairment, we found no evidence of viral replication, as shown by stable or decreasing viral loads over time and the lack of detectable replicative viral strands. This suggests that fitness costs arise from host immunopathology rather than active infection [52]. One plausible mechanism is immune-mediated self-damage: ABPV particles may trigger antiviral defences such as apoptosis and subsequent phagocytosis, but excessive apoptosis near vital organs can harm the host [53,54]. Similarly, activation of the phenoloxidase cascade and the release of cytotoxic intermediates or reactive oxidative species confer broad antimicrobial activity, but can cause host tissue damage and early senescence [55–59].

We speculate that the lack of ABPV replication in *O. biroi* is due to host specificity limiting viral infectivity and pathogenicity in a novel host [20,60]. Host phylogeny strongly predicts infection outcomes, as shown for *Drosophila* C virus and Cricket paralysis virus, where changes in viral load correlate with relatedness between natural and novel hosts [61,62]. Successful infection typically requires compatible host cell surface receptors for viral attachment and entry (Maginnis et al., 2018; Weaver et al., 2021) and ABPV may lack the receptor-binding domains required to interact with *O. biroi* cells. Additionally, viral host shifts are often constrained by host-specific physiological and immune barriers, requiring substantially higher inoculation doses to overcome immunity and initiate replication in a novel host [19,33,63,64], a threshold potentially not reached in our experiments. Taken together with cross-species infection experiments in ants [33,65] and bumblebees [19,49,63], our results indicate that although honey bee viruses can spill over into novel hosts and impair fitness, onwards interspecific transmission or spillback to honey bees is not guaranteed and greatly depends on exposure dosage and host-virus compatibility [27,28].

Consistent with the absence of replication, *Dcr-2* expression was not induced by ABPV injection, indicating no activation of the RNAi antiviral pathway. Both ABPV- and control-injected ants showed similar temporal shifts in *Dcr-2* expression, likely reflecting a general response to injection-induced wounding rather than virus-specific immunity. Alternatively, other immune pathways might be involved, as insect antiviral responses can vary across species [66,67].

Adult ants cohabiting with ABPV-injected pupae or adults acquired detectable viral loads within one to three days. Transmission likely occurred through social contact, such as mutual grooming and brood care, or via environmental contamination of the shared nest space [63,68]. Infected carcasses may also have contributed to viral uptake by cohabiting individuals, as honey bee viruses can remain viable and infectious after host death[69]. Other possible uptake

routes include the consumption of pupal fluid [70] or brood cannibalism, which we observed in our experiment and is used by other ants to reduce infection risk [71]. The unexpectedly high viral loads in cohabiting adults may therefore reflect repeated recontamination or incomplete immune clearance, similar to the observation that direct contact and cannibalism facilitate efficient intraspecific virus transmission [72].

Our work adds to evidence (Valizadeh et al., 2025) showing that ants can acquire and carry bee viruses without supporting active replication. Their role as incidental hosts or mechanical reservoirs may facilitate viral maintenance and transmission across insect communities [73]. Positive-sense RNA viruses, such as ABPV, replicate rapidly and with high error rates, which can accelerate their adaptation to novel hosts [9]. Consequently, even transient uptake by ants may promote low-level viral persistence that, under selective pressure, may give rise to variants capable of overcoming interspecific barriers and initiating host shifts, a process recognised as a key driver of emerging infectious diseases [60]. Environmental stressors, such as pesticide exposure [74,75] and climate change [76], which affect host immune function and physiology, can potentially amplify the risk of adaptation to novel hosts and cross-species transmission of bee viruses.

Our findings highlight the potential of honey bee viruses to impose fitness costs and transmit in non-Apis hosts, such as ants, even without active replication. Such hosts can potentially act as reservoirs of infection and contribute to future spillover or spillback events, thereby shaping the epidemiology of bee pathogens in insect communities. Understanding these dynamics is critical for predicting viral emergence and designing strategies to safeguard pollinator health and enhance ecosystem resilience.

Author contributions:
L.K.L.: conceptualisation, experimental design, data collection and analysis, manuscript writing, manuscript reviewing, and editing; A.B.: experimental design, data collection and analysis, manuscript reviewing; R.J.P.: conceptualisation, manuscript reviewing and editing, funding; Y.U.: conceptualisation, manuscript writing, reviewing and editing, funding.

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The authors declare no competing interests.

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